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Short communication

Inhaled delivery of Interferon-lambda restricts epithelial-derived Th2 inflammation in allergic asthma

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ABSTRACT

The possibility has been suggested that interferon (IFN)-λs can be induced rapidly for restricting respiratory viral infection in asthmatic mice and may modulate Th2-related immune responses that underlie the pathogenesis of asthma. We sought to determine the *in vivo* contribution of IFN-λs on decrease of Th2 cytokines in the respiratory tract of *in vivo* asthma. Lungs of asthmatic mice were severely inflamed, with extensive inflammatory cell infiltration and increased goblet cell metaplasia with higher total lung resistance. The mean protein levels of TSLP and IL-33 from BAL fluid of asthmatic mice were significantly higher until 7 days. Following the collection of lung tissue of 20 asthmatic mice, TSLP and IL-33 gene expressions inversely correlated with mRNA levels of IFN-$\lambda_{2/3}$. Asthmatic mice were administered recombinant IFN-$\lambda_{2/3}$ via the intranasal route and the mRNA levels of IFN-stimulated genes were elevated to an even greater extent in the lung tissue of the mice without intranasal IFN-$\lambda_{2/3}$. Asthma-related histopathologic lung inflammation was significantly improved and total lung resistance was maintained within normal range in IFN-$\lambda_{2/3}$-treated asthmatic mice. Moreover, IFN-$\lambda_{2/3}$-treated asthmatic mice exhibited significant decrease of secreted protein levels of TSLP and IL-33 in the BAL fluid until 7 days after IFN administration. The current data provide compelling evidence that the compensation of IFN-λs can restrict the secretion of epithelial-derived Th2 cytokines, accompanied with reduced asthmatic immunopathology and IFN-λs are critical for limiting Th2-mediated allergic responses in allergic asthma.

1. Introduction

Allergic asthma is thought to arise from an imbalance in T helper type I (Th1)-Th2 immune regulation, resulting in increased levels of the Th2 cytokines interleukin (IL)-4, IL-5, and IL-13 [1]. Asthma exacerbations are acute attacks of asthma when an otherwise stable asthmatic experiences a rapid increase in symptoms accompanied by decreased lung function, most often precipitated by a respiratory viral infection [2]. Asthma exacerbations are responsible for the vast majority of the morbidity and mortality associated with asthma. Although adequate control of asthma has been achieved, better treatments are needed to reduce acute exacerbation of respiratory symptoms in asthmatics.[2]

The innate immune system of the respiratory epithelium serves as the first line of antiviral defense against invading respiratory pathogens and inhaled allergens and this system culminates in the production of interferon (IFN), a key molecule in the innate immune response. Several studies have documented abnormal immune responses to pathogens in patients with allergic asthma and dysregulation of innate immune responses against respiratory infection has been suggested to explain the higher aggravation of Th2-related inflammation of the asthmatic respiratory epithelium to allergens [3]. Strong links between low expression of type I IFNs (IFN-α and –ß), severity of allergic asthma or asthma exacerbations have been described and asthmatic patients with active disease still exhibit an inverse correlation between IFN–ß level and the severity of allergic response in the airways [3].

Recent studies showed the possibility that administration of IFNs ameliorates the asthma-related pathologic findings and Th2-induced inflammation that occur in mice after sensitization and challenge with allergens model can be reduced *in vivo* asthma with exogenous IFNs treatment [4]. Moreover, IFN–ßs might have additional properties beyond their antiviral capacity, such that they are possible antagonists of Th2-induced immune responses [5].

However, our previous study showed that IFN-λs are more predominant IFNs produced in the mouse respiratory tract to resist respiratory viral infection through enhancing the innate immune response to acute lung infection [6]. Our findings also implicated that intranasal

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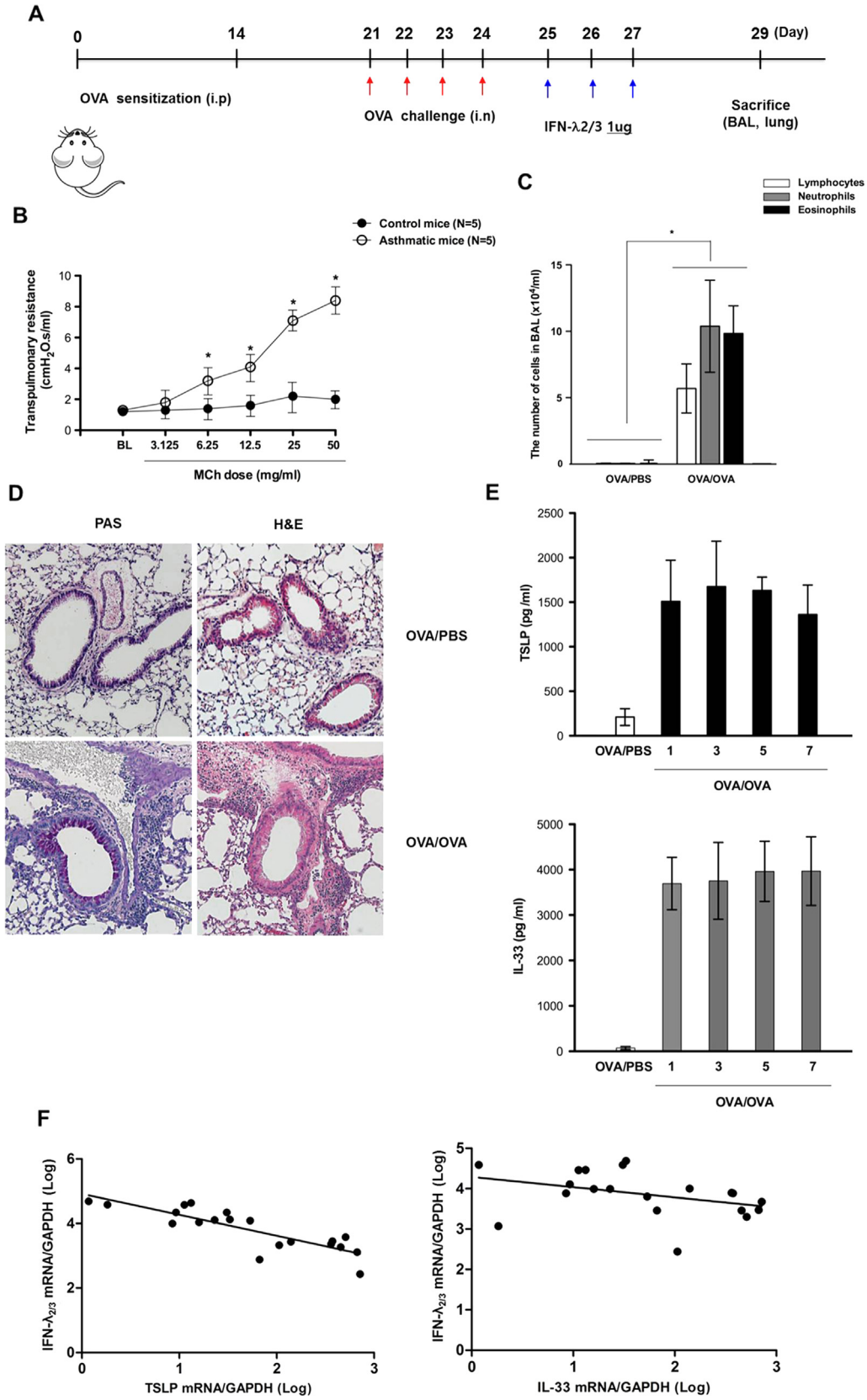

Fig. 1. The development of allergic asthmatic mouse model and the expression of epithelial-derived Th2 cytokines. (A) The experimental protocol for development of allergic asthmatic mouse using C57BL/6. (B) Airway hyper-responsiveness was measured as methacholine-induced increases in total lung resistance (RL) in mechanically ventilated mice. Data are expressed as mean value of percentage increased from base line of the total RL ± standard deviation (SD) of five mice (White dot: OVA/OVA, Black dot:OVA/PBS). (C) BAL fluid differential counts for lymphocytes, neutrophils and eosinophils expressed as mean ± SD. (D) Histological assessment of mucus secretion in asthmatic mice. Periodic acid Schiff (PAS)-stained sections were assessed from five mice. (E) ELISA assay was performed to quantify the levels of secreted Th2 cytokines (i.e., TSLP and IL-33) in the BAL fluid of non-asthmatic and asthmatic mice until 7 days. (F) IFN-$\lambda_{2/3}$ gene expression in the lung tissue of asthmatic mice (OVA/OVA) was normalized, log transformed and correlated with gene expression of TSLP and IL-33. The data is presented as dot plots with fitted regression lines. Significant Spearman R-value is indicated (IFN-$\lambda_{2/3}$ mRNA vs TSLP: r = -0.8361, p = 0.018, IFN-$\lambda_{2/3}$ mRNA vs IL-33: r = -0.4575, p = 0.049).

application of IFN-λs may be able to control acute lung infection, regardless of the effect of IFN-α and –ß [6]. In addition, IFN-λs could be induced rapidly in asthmatic mice after IAV infection, and that this cytokine is important for restricting Th2 cytokine secretion in asthmatic mice [4]. These findings suggest the possibility that IFN-λs may modulate Th2-related immune responses that underlie the pathogenesis of asthma and can have therapeutic potential for reducing Th2 cytokines in asthmatics. To address these issues, we sought to determine the *in*

vivo contribution of IFN-λs on decrease of Th2 cytokines in the respiratory tract of asthma and found that the intranasal delivery of IFN-λs led to accelerated reduction of TSLP and IL-33, accompanied by improved Th2-mediated histopathologic findings in the lung of asthmatic mouse.

2. Material and method

Additional methodological details are available in the online supplement

2.1. Ethics statement

All experiments were approved by the Institutional Review Board of Seoul National University College of Medicine (IRB number 2015-2642) and were carried out in accordance to LABORATORY ANIMAL ACT of Korean Ministry of Food and Drug Safety for enhancing the ethics and reliability on animal testing through appropriate administration of laboratory animals and animal testing.

2.2. Allergen sensitization and challenge protocol

C57BL/6J (B6) mice (Orientalbio, Seoul, Korea) aged 7 weeks (19–23 g) were used for development of non-asthmatic and asthmatic mice. Asthma was induced by first sensitizing male B6 mice intraperitoneally (i.p.) with OVA in aluminum hydroxide and then challenging intranasally (i.n.) with soluble OVA (OVA/OVA). PBS-challenged mice (OVA/PBS) (hereafter referred to as non-asthmatic mice) were used as a negative control. Airway hyper–responsiveness (AHR) was measured in anesthetized mechanically ventilated B6 mice (Flexivent ventilator, SciReq, Montreal, Canada) at 24 h after the last intranasal OVA exposure. AHR was measured invasively using a body plethysmograph (Buxco Electronics, Inc, Wilmington, NC, USA).

2.3. Real-time PCR

Lung tissue was obtained from asthmatic mice on 1, 3, 5, and 7 days post of OVA challenge, after which total RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized from 3 μg of RNA with random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Life Sciences, Waltham, MA, USA and Roche Applied Science, Indianapolis, IN, USA). Target mRNA levels were quantified using target-specific primer and probe sets for mouse IFN-$\lambda_{2/3}$ (Mm04204155_gH), TSLP (Mm01157588_m1), IL-33 (Mm00505403_m1), CXCL10 (Mm00445235_m1), IFIT2 (Mm00492606_m1), Mx1 (Mm00487796_m1), and OAS2 (Mm00460961_m1). All PCR assays were quantitative and utilized plasmids containing the target gene sequences as standards.

2.4. Quantification of secreted cytokines

The levels of secreted TSLP (DY555) and IL-33 (DY3626) were quantified using a Duoset ELISA kit (R&D Systems; Minneapolis, MN, USA) according to the manufacturer's instructions for BAL fluid.

2.5. Intranasal delivery of recombinant IFN-λ2/3

To determine whether IFN-$\lambda_{2/3}$ controls Th2 inflammation in the respiratory tract of *in vivo* asthma model, asthmatic mice were exposed to whole body mainstream by the SCIREQ "InExpose" system (SCIREQ, Montreal, QB, Canada) and recombinant IFN-$\lambda_{2/3}$ (total volume of 30 ul, IFN-λ_2: 1 ug, IFN-λ_3: 1 ug) (Invitrogen, Carlsbad, CA, USA) was inoculated into asthmatic mice three times by intranasal delivery (Fig. 1a).

2.6. Immunohistochemistry and histologic analysis

Lung tissue was fixed in 10% (vol/vol) neutral buffered formalin and embedded in paraffin. Paraffin-embedded tissue slices were stained with hematoxylin/eosin (H&E) or periodic acid–Schiff (PAS) solution (Sigma, Deisenhofen, Germany). Histopathologic analysis of inflammatory cells in H&E and PAS stained lung sections was performed in a blinded fashion using a semi-quantitative scoring system as previously described [4].

2.7. Statistical analyses

Real-time PCR and ELISA results are presented as median values (interquartile ranges for 25% and 75%). The statistical significance of differences between two groups was determined by the Mann-Whitney test. Total lung resistance was also evaluated using a non-parametric test (Wilcoxon rank sum test) and the statistical correlation between TSLP, IL-33 and IFN-$\lambda_{2/3}$ was analyzed through Spearman's correlation analysis. All statistical analysis was performed with GraphPad Prism software (version 5; GraphPad Software, La Jolla, CA, USA). P values < 0.05 were considered to be statistically significant.

3. Results

3.1. A typical asthmatic phenotype of OVA-sensitized/challenged C57BL/6 (B6) mice

We found that asthmatic mice (OVA/OVA) were observed to have a significant methacholine-induced increase in total lung resistance (N = 5, Fig. 1b) and the numbers of lymphocytes, neutrophils, and eosinophils were significantly elevated in the BAL fluid of asthmatic mice (Fig. 1c). As a complementary approach, H&E- and PAS-stained micrographs of lung sections were obtained from non-asthmatic (OVA/PBS) and asthmatic mice. Histological analysis revealed that the lungs of asthmatic mice were severely inflamed, with extensive inflammatory cell infiltration at the peribronchial areas of the lung. This infiltration was accompanied by significantly increased goblet cell metaplasia (Fig. 1d). The results of ELISA showed that the mean protein levels of epithelial-derived Th2 cytokines including TSLP and IL-33 from BAL fluid of asthmatic mice were significantly higher until 7 days (Fig. 1e). As a next step, we investigated the correlation between epithelial-derived Th2 cytokines and IFN-λ mRNA levels in the lung tissue of asthmatic mice. Following the collection of lung tissue of 20 asthmatic mice (OVA/OVA), TSLP, IL-33, and IFN-$\lambda_{2/3}$ mRNA levels were measured using the cell lysate of lung tissue. Both TSLP ($r = -0.8361$, $p = 0.018$) and IL-33 ($r = -0.4575$, $p = 0.049$) gene expressions inversely correlated with mRNA levels of IFN-$\lambda_{2/3}$ in the lung of asthmatic mice (Fig. 1f). Collectively, these findings demonstrate that an allergic asthma mouse model could be established with B6 mice, and that this model could be used to investigate the therapeutic potentials of inhaled IFN-λs for reduction of epithelial-derived Th2 cytokines in asthmatic mice.

3.2. Therapeutic blockade of epithelial-derive Th2 cytokines through inhalation of IFN-λs

In the present study, we focused on new insight about the role of type III IFNs for reducing epithelial-derived Th2 cytokines in this asthmatic airway and measured the secreted protein levels of TSLP and IL-33 in asthmatic mice until 7 days after development of asthma. To comprehensively examine the type III IFN-induced innate immune responses, asthmatic mice (N = 5) were administered recombinant IFN-$\lambda_{2/3}$ via the intranasal route and the mRNA levels of ISGs (CXCL10: 1.2×10^3, IFIT2: 2.2×10^3, dMx1: 1.9×10^3, OAS2: 3.8×10^3) that are required for IFN-stimulated the innate immune response were elevated to an even greater extent in the lung tissue of the mice that

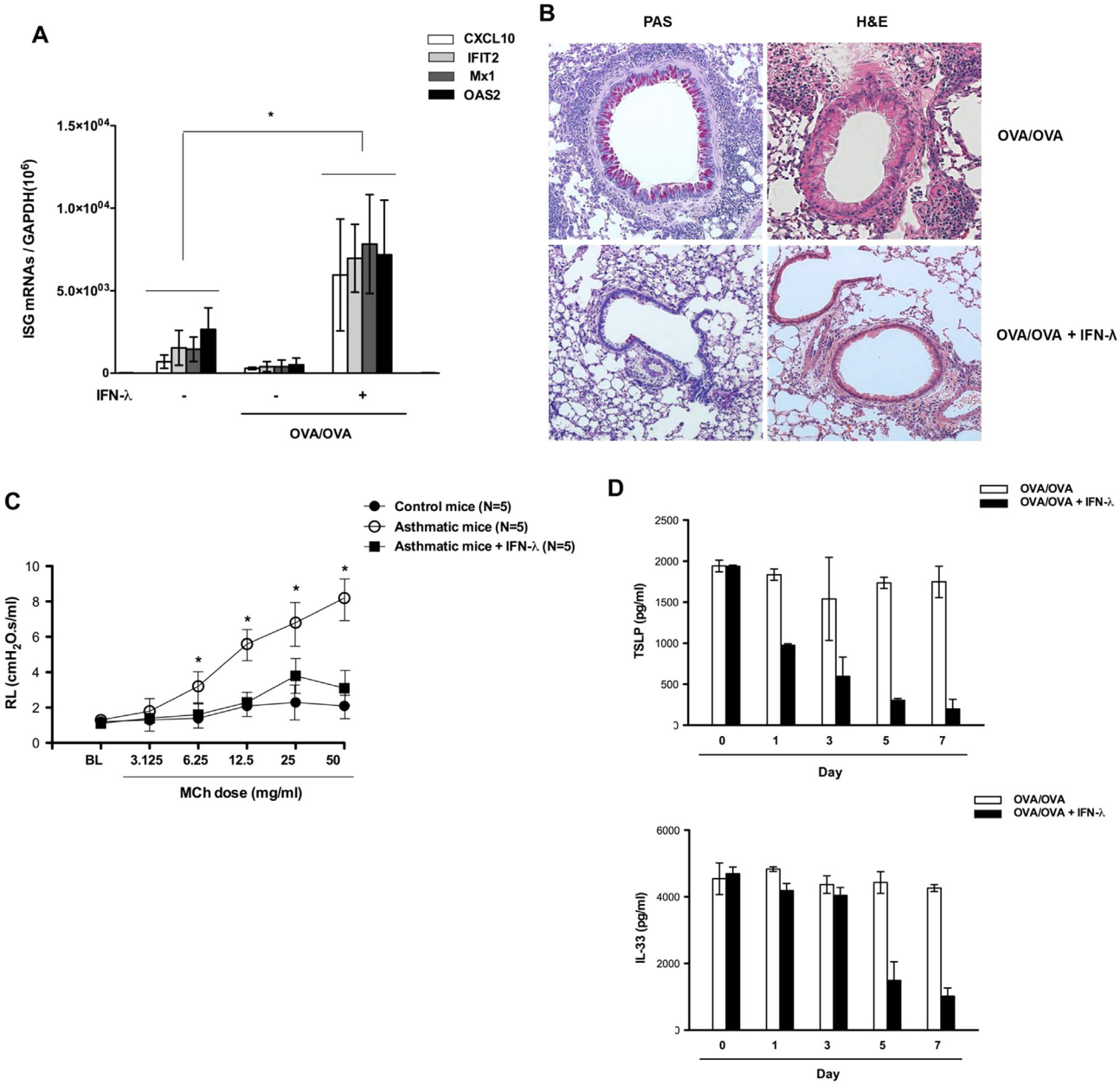

Fig. 2. Kinetics of IFN-λ-inoculated asthmatic mice. Asthmatic mice (OVA/OVA, N = 5) were exposed to whole body mainstream by the SCIREQ "InExpose" system and recombinant IFN-$\lambda_{2/3}$ (total volume of 30 ul, IFN-λ_2: 1 ug, IFN-λ_3: 1 ug) were inoculated to asthmatic mice. (A) mRNA levels of IFN-stimulated genes such as CXCL10, IFIT2, Mx1, and OAS2 were measured using real-time PCR. (B) Histological assessment for Hematoxylin and eosin (H&E)- and PAS-staining lung sections of asthmatic mice was performed after recombinant IFN-λ inoculation by intranasal delivery. (C) Total lung resistance was determined in recombinant IFN-λ-inoculated asthmatic mice and data are expressed as mean value of percentage increased from base line of the total RL ± standard deviation (SD) of five mice (White dot: OVA/OVA, Black dot:OVA/PBS). (D) ELISA assay was performed to quantify the levels of secreted TSLP and IL-33 levels in the BAL fluid of recombinant IFN-λ-inoculated asthmatic mice. Micrographs shown are representative of lung sections from five mice. PCR, plaque assay, and multiplex assay results are presented as mean ± SD from five independent experiments (*, $p < 0.05$ compared with the levels of IFN-λs-inoculated asthmatic mice).

received intranasal IFN-$\lambda_{2/3}$ (CXCL10: 6.2×10^4, IFIT2: 6.8×10^4, dMx1: 7.8×10^4, OAS2: 7.1×10^4, Fig. 2a).

To determine whether inhaled administration of IFN-$\lambda_{2/3}$ reduces Th2 inflammation in asthmatic airway, asthmatic mice (N = 5) were administered recombinant IFN-$\lambda_{2/3}$ via the intranasal route. Interestingly, IAV-mediated histopathologic lung inflammation was significantly improved and methacholine-induced increase of total lung resistance was not observed in IFN-$\lambda_{2/3}$-treated asthmatic mice (Fig. 2b and c). Moreover, IFN-$\lambda_{2/3}$-treated asthmatic mice exhibited significant decrease of secreted protein levels of TSLP and IL-33 in the BAL fluid until 7 days after IFN-$\lambda_{2/3}$ administration (Fig. 2d). The current findings support the idea that significant induction of epithelial-derived Th2 promoting cytokines in asthmatic mice could be suppressed through inhaled administration of IFN-λs although strong correlation between induction of epithelial-derived Th2 cytokines and lower expression of IFN-λs was observed in the airway of asthmatic mice.

4. Discussion

The present study provides a distinctive role of IFN-related innate immune system for limitation of Th2-mediated allergic responses in the asthmatic airway and the significant reduction of epithelial-derived Th2 cytokines through the inhaled delivery of IFN-λs. Here, we showed that inhaled administration of IFN-$\lambda_{2/3}$ led to restrict Th2 cytokine secretion

such as TSLP and IL-33 in asthmatic mice and our findings also imply that exogenous compensation of IFN-λs is a potential strategy for controlling the Th2-mediated allergic responses of asthmatic mice at the level of respiratory epithelium.

Asthma is a heterogenous inflammatory disorder of the lung caused principally by sensitization and exposure to an allergens and asthma exacerbation is responsible for vast majority of the morbidity and mortality associated with asthma [1,2]. To the best of our knowledge, deficient innate immune responses (including reduced induction of IFNs) are expected in the asthmatic respiratory tract [3] and the experiments performed here also showed that asthmatic mice exhibited a significantly induction of TSLP and IL-33 in the BAL fluid, accompanied by impaired production of IFN-λs. Additional research aiming to understand the mechanisms driving these deficient innate immune responses is urgently required to reduce the asthma exacerbation and such studies are necessary for identifying novel targets for the development of effective therapies against Th2-related inflammation in asthmatics [7,8]. Our study focused on the influence of IFN-related innate immune responses in respiratory epithelium to suppress Th2-mediated allergic responses in asthma and we found that inhaled delivery of IFN-λs led to reduced Th2-related immune responses, including decreased TSLP and IL-33 secretion. Moreover, resolution of extensive asthma-related lung pathologies was observed and metacholline-induced total lung resistance was completely improved in IFN-λs -treated asthmatic mice.

We speculated that the therapeutic effect of IFN-λs against allergic responses is restricted to the upper and lower respiratory tracts, which contain high percentages of epithelial cells that express the IFN-λ receptor [9]. Therefore, the inhaled delivery of IFN-λs and induction of IFN-stimulated genes' transcription more effectively suppressed epithelial-derived Th2 cytokines secretion in asthmatic respiratory tract. Recently, IFN-λs were also found to act on non-hematopoietic cells to control viral infection and the regulation of adaptive immune response *in vivo* lung tissue, indicating that the anti-inflammatory mechanism against Th2 cytokines of IFN-λs is direct to the respirator epithelium of asthmatic lung [4]. In addition, the aggravation of asthma increases might be exponentially related with a higher risk of respiratory viral infection and is a leading cause of the morbidity and mortality in asthmatics [2]. Our data suggest the possibility that the treatment of IFN-λs and induction of ISGs can control acute viral lung infection in asthmatics and IFN-λs are also a promising new target to prevent the risk of exacerbation in asthma or higher mortality of asthmatics.

Taken together, the current data provide compelling evidence that the compensation of IFN-λs can restrict the secretion of epithelial-derived Th2 cytokines, accompanied with reduced asthmatic immunopathology and IFN-λs are critical for limiting Th2-mediated allergic responses in allergic airway disease. Therefore, strategies involving the compensation or maintenance of IFN-λs are new opportunities for invoking an effective control of allergic asthma.

5. Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.02.010.

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METHODS

Ethics statement

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Real-time PCR

Lung tissue was obtained from asthmatic mice on 1, 3, 5, and 7 days post of OVA challenge, after which total RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized from 3 μg of RNA with random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Life Sciences, Waltham, MA, USA and Roche Applied Science, Indianapolis, IN, USA). Amplification was performed using the TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Briefly, amplification reactions had a total volume of 12 μl and contained 2 μl of cDNA (reverse transcription mixture), oligonucleotide primers (final concentration of 800 nM), and TaqMan hybridization probe (200 nM). Real-time PCR probes were labeled at the 5' end with carboxyfluorescein (FAM) and at the 3' end with the quencher carboxytetramethylrhodamine (TAMRA).

To quantify the intracellular levels of host gene expression levels, cellular RNA was used to generate cDNA. IAV level was monitored using quantitative PCR. Primers for mouse IFN-$\lambda_{2/3}$, TSLP, IL-33, CXCL10, IFIT2, Mx1, and OAS1 were purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed using the PE Biosystems ABI PRISM® 7700 Sequence Detection System. Thermocyling parameters were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Target mRNA levels were quantified using target-specific primer and probe sets for mouse IFN-$\lambda_{2/3}$ (Mm04204155_gH), TSLP (Mm01157588_m1), IL-33 (Mm00505403_m1), CXCL10 (Mm00445235_m1), IFIT2 (Mm00492606_m1), Mx1 (Mm00487796_m1), and OAS2 (Mm00460961_m1).

All PCR assays were quantitative and utilized plasmids containing the target gene sequences as standards. All reactions were performed in triplicate, and all real-time PCR data were normalized to the level of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH, 1×10^6 copies) to correct for variation between samples.

Quantification of secreted cytokines

The levels of secreted TSLP (DY555) and IL-33 (DY3626) were quantified using a Duoset ELISA kit (R&D Systems; Minneapolis, MN, USA) in BAL fluid according to the manufacturer's instructions. This kit detects TSLP and IL-33 protein levels which are secreted to BAL fluid in wild type and asthmatic mice. The working range of the assay was 62.5-4000 pg/ml. To quantify the secreted proteins of TSLP and IL-33, we used recombinant mouse TSLP & IL-33 Standard included within the Duoset and a seven point standard value from 15.6 to 1,000 pg/ml using 2-fold serial dilutions in Reagent Diluent.

Intranasal delivery of recombinant IFN-$\lambda_{2/3}$

To determine whether IFN-$\lambda_{2/3}$ controls Th2 inflammation in the respiratory tract of *in vivo* asthma model, asthmatic mice were exposed to whole body mainstream by the SCIREQ "InExpose" system (SCIREQ, Montreal, QB, Canada) and recombinant IFN-$\lambda_{2/3}$ (total volume of 30 ul, IFN-λ_2: 1 ug, IFN-λ_3: 1 ug) (Invitrogen, Carlsbad, CA, USA) was inoculated into asthmatic mice three times by intranasal delivery (Fig 1A).

Immunohistochemistry and histologic analysis

Lung tissue was fixed in 10% (vol/vol) neutral buffered formalin and embedded in paraffin. Paraffin-embedded tissue slices were stained with hematoxylin/eosin (H&E) or periodic acid–Schiff (PAS) solution (Sigma, Deisenhofen, Germany). Histopathologic analysis of inflammatory cells in H&E-stained lung sections was performed in a blinded fashion using a semi-quantitative scoring system as previously described [4]. Lung sections from at least five mice were examined. Briefly, peribronchiolar inflammation was scored as follows: 0, normal; 1, a few cells; 2, a ring of inflammatory cells one layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells more than four cells deep (maximum score=8). The histological score for PBS/PBS control mouse lung tissue was 0. At least six separate areas from similar sections within a single mouse were assessed, and at least five mice were assessed. The five best sections were used for evaluation. PMNs were counted by an examiner who was blinded to the experimental group; results are expressed as the number of cells per high power field. Morphometric analysis of PAS stained sections was performed by quantifying PAS pixels per μm length distance of bronchial epithelium of central airways using the Image J software. At least six areas from similar sections per mouse and at least six mice were assessed blindly. PAS score for PBS/PBS control mouse lungs was constantly <2.

Differential cell counting of BAL fluid

On day 29 after establishment of asthma, the animals were anesthetized with ketamine plus xylazine, sacrificed, and their lungs were flushed for the total leukocyte and differential cell counts. BAL (1ml) was collected in an Eppendorf tube from each mouse under anesthesia and used for leukocyte count. The way that we

were counting the number of immune cells in BAL fluid of each mouse in the present study was diff quick staining method (DQ, International Reagents Co., Ltd., Kobe, Japan) to identify eosinophils, lymphocytes, macrophages or neutrophils using standard morphologic determinants. DQ preparations were obtained from an aliquot of BAL fluid. A thin smear of each BAL fluid was stained by the DQ method according to the data sheet recommendations. Briefly, the air dried smears were sequentially dipped five times in a methanol fixative solution, five more times in stain solution I (eosin G), then dipped five times in stain solution II (thiazine dye) and, finally, gently rinsed with distilled water. The overall staining process took about 15 seconds. The slides were then cleared in xylol and coverslipped with a non-aqueous mounting medium. Moreover, an aliquot of each BAL fluid was diluted with normal saline to obtain a final leucocyte concentration of 400 cells/ml. One hundred microlitres of the cell suspension were cytocentrifuged onto two degreased slides, air dried and, finally, stained by the DQ method. A DQ stained smear of each BAL fluid was blindly analyzed. Analysis was carried out using ´400 magnification and, if necessary, ´1000 magnification with an oil objective (Leika DMRB microscope).

Statistical analyses

Real-time PCR and ELISA results are presented as median values (interquartile ranges for 25% and 75%). The statistical significance of differences between two groups was determined by the Mann-Whitney test. Total lung resistance was also evaluated using a non-parametric test (Wilcoxon rank sum test) and the statistical correlation between TSLP, IL-33 and IFN-$\lambda_{2/3}$ was analyzed through Spearman's correlation analysis. All statistical analysis was performed with GraphPad Prism

software (version 5; GraphPad Software, La Jolla, CA, USA). P values <0.05 were considered to be statistically significant.